BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Docket No: C38435/109700CON

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :)

Akira ASAKURA *et al.*) Examiner: M. Walicka

Serial No.: 09/470,667) Art Unit: 1652

Filed: December 22, 1999)

For: **NOVEL ALCOHOL/ALDEHYDE DEHYDROGENASES**)

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF MR. MASAO MASHITA UNDER 37 C.F.R. § 1.132

Sir:

I, Masao Mashita, a citizen and resident of Japan, hereby declare as follows:

1. I am employed by Sawady Technology Co., Ltd., 1-29-10, Maeno-cho, Itabashi-ku, Tokyo, Japan 174-0063 ("Sawady"). I hold the position of Sales & Marketing Director at Sawady. One of my duties at Sawady is to coordinate nucleic acid sequencing orders for clients of Sawady.

2. By way of background, Sawady is an independent commercial entity that is not affiliated with the Nippon Roche Research Center of Nippon Roche K.K. ("NRKK"). Among the services provided by Sawady to its clients is the sequencing of nucleic acid molecules.

It is in this capacity that I was initially contacted by Dr. Masako Shinjoh of NRKK regarding Sawady's ability and interest in sequencing a certain part of the chromosomal DNA derived from *Gluconobacter oxydans* DSM 4025 as set forth in more detail below.

3. As a follow up to my discussions with Dr. Shinjoh, on August 8, 2000, I was asked by Mr. Naoki Itoh, NRKK's Patent & Licensing, Manager, to have Sawady sequence certain portions of the chromosomal DNA of *Gluconobacter oxydans* DSM 4025 using two pairs of primers identified by NRKK. On the same day, Mr. Itoh forwarded to me via e-mail (i) an order letter, (2) an order form, and (3) general protocols to be used in the sequencing. A copy of Mr. Itoh's e-mail (and attachments) is attached as Exhibit 1 and an English translation of the e-mail is attached as Exhibit 2.

4. On August 11, 2000 I received an e-mail from Dr. Shinjoh (original written in Japanese attached as Exhibit 3; its English translation as Exhibit 4) indicating that she would send to me, via overnight courier, a package containing an ampoule of lyophilized cells of *Gluconobacter oxydans* DSM 4025 on August 16, 2000.

5. On the morning of August 17, 2000, I received the package referenced in Dr. Shinjoh's August 11, 2000 e-mail.

6. As is our standard practice, I engaged Mr. Yoshitaka Murata of K.K. Kyurin Corporation (27-25, Morishita-cho, Yahatanishi-ku, Kitakyushu-shi, Fukuoka-ken, 806-0046 Japan) ("Kyurin") to reconstitute the lyophilized cells in the ampoule I received from Dr. Shinjoh, to grow up those cells, and to isolate chromosomal DNA from those cells. Specifically, on August 17, 2000, I sent the ampoule I received from Dr. Shinjoh to Mr. Murata's company together with an instruction letter requesting that Mr. Murata provide me with isolated chromosomal DNA from the lyophilized cells in the ampoule. A copy of our order letter to Kyurin is attached as Exhibit 5 (in Japanese) and its English translation is attached as Exhibit 6.

7. On August 18, 2000, I received, confirmation that Mr. Murata received the ampoule and order letter.

8. On September 1, 2000, I received a package from Mr. Murata containing chromosomal DNA isolated from *Gluconobacter oxydans* DSM 4025 cells reconstituted from the lyophilized cells contained in the ampoule I sent to Mr. Murata. *See* the copy of the DECLARATION OF YOSHITAKA MURATA UNDER 37 C.F.R. §1.132 attached as Exhibit 7 (without Exhibits).

9. Using the isolated DNA forwarded from Mr. Murata, I supervised the nucleotide sequencing conducted by Mr. Susumu Yamashita at

Sawady in accordance with the instructions of Dr. Shinjoh, the results of which are reported in the Experimental Report attached as Exhibit 8 (in Japanese) and its English translation as Exhibit 9.

10. The Experimental Report (non-finalized) was forwarded to Dr. Shinjoh on October 10, 2000 via Mr. Itoh. The Experimental Report was finalized and executed on September 12, 2002.

11. In sum, the ampoule containing the lyophilized *Gluconobacter oxydans* DSM 4025 cells that I received from Dr. Shinjoh on August 17, 2000 was the same ampoule that I forwarded to Mr. Murata at Kyurin on the same day I received it. And, the chromosomal DNA I received from Mr. Murata on September 1, 2000 was the same DNA that was used in the sequencing experiments performed under my supervision at Sawady, the results of which are reported in Exhibits 8 and 9.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12. Sep 2002

Masao Mashita

Shinjoh, Masako (NRRC~Tokyo)

差出人: Itoh, Naoki (CPO~Tokyo)
送信日時: 2000年8月8日火曜日 午前 11:50
宛先: 'sales@sawady.com'
CC: Shinjoh, Masako (NRRC~Tokyo)
件名: 遺伝子配列決定依頼の件

（株）サワディー・テクノロジー
営業担当取締役
間 下 正 雄 様

お世話になっております。
先日は、ご多忙のところ打合わせのお時間を戴きまして有難うございました。
打合わせにおいて申し上げました以下の書面をお送りしますので、内容ご確認ください。
(1) Order Letter
(2) ORDER FORM（日本語の正本、及びその英訳）
(3) GENERAL PROTOCOLS（英文の正本、及びその日本語訳）

試料を、打合わせに従いまして８月１６日に発送いたします（１７日御社着）ので、上記書面に訂正の必要がありましたら至急にお知らせください。

以上、宜しくお願い申し上げます。

伊藤直樹
日本ロシュ（株）
ライセンシング＆パテントグループ

SAWADY-1.doc SW-ORDERFORM-1.doc SW-ORDERFORM-JP.doc SW-GEN-PRO.doc SW-GEN-PRO-JP.doc

Declaration MMashita
Exhibit-1

4/26

46/70

[T2] E-mail from Mr. Itoh to Mr. Mashita dated Aug. 8, 2000

From: Itoh, Naoki

Date: August 8, 2000 11:50 am

To: Sales@sawady.com

cc: Shinjoh, Masako {NRRC~Tokyo}

Subject: Determination of nucleotide sequence

K. K. Sawady Technology
Sales Marketing Director
Mr. Masako Mashita,

Thank you for your usual service and taking a time for us in your busy schedule the other day.
Please confirm the following papers I explained at that time
(1) Order Letter
(2) ORDER FORM (Original in Japanese, Translation in English)
(3) GENERAL PROTOCOLS ((Original in English, Translation in Japanese)
According to our discussion, we will send the sample on Aug. 16 (it will arrive at your company on Aug. 17). If you have any amendment for the papers above, let us know as soon as possible.

Best regards,
Naoki Itoh
Nippon Roche K.K.
Licensing&Patent group

Attached files:

<SAWADY-A.doc>; <SW-ORDERFORM-1.doc>; <SW-ORDERFORM-JP.doc>
<SW-GEN-PRO.doc>; <SW-GEN-PRO-JP.doc>

Declaration M Mashita
Exhibit-2

5/26

47/70

Shinjoh, Masako {NRRC~Tokyo}

差出人: Shinjoh, Masako {NRRC~Tokyo}
送信日時: 2000年8月11日金曜日 午後 3:53
宛先: 'Sales@sawady.com'
CC: Itoh, Naoki {CPO~Tokyo}
件名: PCR-sequencing 依頼

間下さん

いつもお世話になります。
今回の、sequencingもよろしくお願いいたします。
注文の詳細は、弊社伊藤がお伝えしましたように、来週水曜日、8月16日に材料とともにクロネコ宅急便で、17日午前到着指定でおくります。

今回は、支払に係る、こちらの注文番号をお伝えいたします。
注文番号: W0005031

それでは、解析の方、よろしくお願いいたします。

新城雅子

日本ロシュ研究所
所属:応用微生物部
氏名:新城雅子
住所: 鎌倉市梶原200
TEL: 0467-47-2226
PAX:0467-45-6812

Declaration M.Mashita
Exhibit -3

フ→E

48/70

6/20

[T3] E-mail from Dr. Shinjoh to Mr. Mashita dated Aug. 11, 2000

From: Shinjoh, Masako

Date: August 11, 2000 3:53 pm

To: Sales@sawady.com

cc: Itoh, Naoki {CPO~Tokyo}

Subject: PCR-sequencing order

Dear Mr. Mashita,

Thank you for your usual service.

I'd like to ask you this sequencing request.

The details of this order and our sample will be sent next Wednesday, Aug. 16, as Mr. Itoh has already let you know via KORONEKO-TAKKYUBIN to arrive to your site in the morning of Aug. 17.

I inform our order number relating the payment;

order number: W0005031.

Could you please perform the analysis.

Best regards,

Masako Shinjoh

NRRC, Applied Microbiology, Masako Shinjoh,

TEL: 0467047-2226 FAX; 0467-45-6812

Declaration M. Mashita
Exhibit-4

7/26

49/50

received
Aug. 27, 2002
M. Shinjoh

Sawady → → → KOLA
KOLA御中

注文書

サワディー管理番号 SW-002

お客様名　新城　様　　平成 12年　8 月　17 日
所属　日本ロシュ研究所
住所　神奈川県鎌倉市梶原200
Tel　　Fax
Email

依頼内容:・菌体よりクロモゾームDNA抽出

納品状態:

Declaration MMashita
Exhibit-5

サンプル: 菌体
DSM4025（凍結乾燥品）
N2寒天培地（2枚）
取り扱い説明書

備考:

その他、連絡事項

KOLA記入欄

予定納期 その他サワディーへの連絡事項等

内容をご確認いただき、ご不明の点はご連絡をいただけますようにお願いいたします。

(株)サワディーテクノロジー
〒171－0022 東京都豊島区南池袋2－9－9 第一池袋ホワイトビル1F
Tel: 03－3988－4633 Fax: 03－3982－5666
Email: product@sawady.com 担当／中川 温子

8/26

50/70

[T5] Order letter from Sawady to Kyurin dated Aug. 17, 2000

Four pages including this page.
Sawady to KOLA

To KOLA

ORDER SHEET

Sawady No. SW-002
Aug. 17, 2000

Client name: Dr. Shinjoh
Organization: Nippon Roche Research Center
Address: 200 Kajiwara Kamakura Kanagawa

Order: Extraction of chromosomal DNA from cells
Shipping form:
Sample: Cells
DSM4025 (Lyophilized)
N2** agar plates
Protocols
Others: Other information
KOLA memo: Planned delivery date Other information to Sawady

Please confirm the items and let us know if you have any questions.

K.K. Sawady Technology
171-0022 Dai-ichi Ikebukuro White Building 1F
2-9-9, Minami-Ikebukuro, Toshima-ku, Tokyo
Tel; 03-3988-4633 Fax; 03-3982-5666
Email:product@sawady.com Atsuko Nakagawa

Declaration
M. Mashita
Exhibit-6

9/26

51/70

Docket No: C38435/109700CON

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :	)	
Akira ASAKURA *et al.*	)	Examiner: M. Walicka
Serial No.: 09/470,667	)	Art Unit: 1652
Filed: December 22, 1999	)	
For: **NOVEL ALCOHOL/ALDEHYDE DEHYDROGENASES**	)	

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF MR. YOSHITAKA MURATA UNDER 37 C.F.R. § 1.132

Sir:

I, Yoshitaka Murata, a citizen and resident of Japan, hereby declare as follows:

1. I am employed by K.K. Kyurin Corporation, 27-25, Morishita-cho, Yahatanishi-ku, Kitakyushu-shi, Fukuoka-ken, 806-0046 Japan ("Kyurin"). I hold the position of Scientist (Kyurin Omtest Laboratory Dept. "KOLA") at Kyurin. One of my duties at Kyurin is to coordinate the preparation of chromosomal DNA from various cell lines in response to orders from clients of Kyurin. A copy of my *curriculum vitae* is attached as Exhibit 1.

2. By way of background, Kyurin is an independent commercial entity that is not affiliated with the Nippon Roche Research Center of

Nippon Roche K.K. ("NRKK"). Among the services provided by Kyurin to its clients is the preparation of chromosomal DNA from various kinds of cell lines. It is in this capacity that I was contacted by Mr. Masao Mashita of Sawady Technology Co., Ltd., 1-29-10, Maeno-cho, Itabashi-ku, Tokyo, Japan 174-0063 ("Sawady") regarding our ability and interest in preparing chromosomal DNA by reconstituting and growing up a lyophilized sample of *Gluconobacter oxydans* DSM 4025 as set forth in more detail below.

3. At the beginning of August, 2000, I was asked by Mr. Mashita to have Kyurin reconstitute, grow up, and harvest chromosomal DNA from a lyophilized sample of *Gluconobacter oxydans* DSM 4025 cells that he would provide to me.

4. On August 10, Mr. Mashita sent a letter to Kyurin via facsimile (a copy of the original facsimile in Japanese is attached as Exhibit 2 and its translation in English is attached as Exhibit 3). This letter confirmed our agreement with Sawady that Kyurin would conduct the requested work and included an "ORDER FORM" (original written in Japanese, a copy of which is attached as Exhibit 4; its English translation is attached as Exhibit 5) and a set of "GENERAL PROTOCOLS" (original written in English, Exhibit 6; its Japanese translation as Exhibit 7) describing the methods to be used by us for isolating the requested chromosomal DNA.

5. On August 18, 2000, I received a package from Mr. Mashita via overnight courier. The package contained an ampoule identified as containing lyophilized cells of *Gluconobacter oxydans* DSM 4025 and an order sheet from Sawady (original written in Japanese, a copy of which is attached as Exhibit 8; its English translation is attached as Exhibit 9).

6. As soon as I received the package, I stored the package in a refrigerator accessible only to authorized Kyurin personnel at 4°C. Later that day, Dr. Sugama, Director, KOLA Kyurin, at my direction, sent an e-mail to Mr. Mashita to confirm receipt of the ampoule and the order letter.

7. On August 26, 2000, I gave Ms. Masako Nomaguchi, a researcher employed by Kyurin, the ampoule I received from Mr. Mashita on August 18, 2000, identified as containing lyophilized cells of *Gluconobacter oxydans* DSM 4025, and instructed Ms. Nomaguchi to reconstitute the lyophilized cells contained in the ampoule, to grow up those cells, and to isolate chromosomal DNA from those cells.

8. On August 31, 2000, Ms. Nomaguchi informed me that she had completed isolating the chromosomal DNA from the cells grown up from the *Gluconobacter oxydans* DSM 4025 sample I had given her, which I had received from Mr. Mashita on August 18, 2000. Ms.

Nomaguchi collected the isolated chromosomal DNA in a 1.5ml tube, which was labeled "SW-2 / DNeasy 28ng / μl 000831 * SW-2 / Sepagene 0.508 μg / μl 000831."

9. On August 31, 2000, I placed the 1.5ml tube labeled "SW-2 / DNeasy 28ng / μl 000831 * SW-2 / Sepagene 0.508 μg / μl 000831" containing the isolated chromosomal DNA prepared by Ms. Nomaguchi into a shipping package. That same day, I forwarded to Mr. Mashita the package containing the tube labeled "SW-2 / DNeasy 28ng / μl 000831 * SW-2 / Sepagene 0.508 μg / μl 000831" containing the chromosomal DNA isolated from the *Gluconobacter oxydans* DSM 4025 cells prepared by Ms. Nomaguchi.

10. In sum, the ampoule containing the lyophilized *Gluconobacter oxydans* DSM 4025 cells that I received from Mr. Mashita on August 18, 2000 was the same ampoule that I gave to Ms. Nomaguchi, who reconstituted the cells, grew them up, and isolated genomic DNA from them. And, the chromosomal DNA that Ms. Nomaguchi isolated from the reconstituted *Gluconobacter oxydans* DSM 4025 cells was the same DNA contained in the tube labeled "SW-2 / DNeasy 28ng / μl 000831 * SW-2 / Sepagene 0.508 μg / μl 000831" that I forwarded to Mr. Mashita on August 31, 2000.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated:_____

_____
Yoshitaka Murata

試験報告書

試験番号 F－903

試験表題：菌株 DSM4025 における遺伝子配列の決定

平成 14 年 9 月 12 日

お客様名：日本ロシュ研究所　日本ロシュ株式会社

神奈川県鎌倉市梶原 200（〒247－8530）

株式会社 サワディー・テクノロジー
東京都板橋区前野町 1 丁目 29 番 10 号（〒174－0063）

試験報告書

表題：

菌株 DSM4025 の遺伝子配列の決定

試験番号：

F-903 (SW-002)

試験委託者

名称：日本ロシュ研究所 日本ロシュ株式会社

所在地：神奈川県鎌倉市梶原 200 (〒247-8530)

試験実施者：

名称：株式会社 サワディー・テクノロジー

所在地：東京都板橋区前野町 1 丁目 29 番 10 号 (〒174-0063)

運営管理者：増尾 正則

試験責任者

氏名：間下 正雄

所属：株式会社 サワディー・テクノロジー

氏名：村田 義隆

所属：株式会社 キューリン

試験担当責任者

氏名：山下 進

所属：株式会社 サワディー・テクノロジー

氏名：野間口 雅子

所属：株式会社 キューリン

実施期間

試験開始日：平成 12 年 8 月 26 日
試験終了日：平成 12 年 9 月 5 日
最終報告書提出日：平成 14 年 9 月 12 日

試料

凍結乾燥菌株
供給源：日本ロシュ研究所 日本ロシュ株式会社

使用試薬および使用機器

この試験を実施するにあたって使用した試薬および機器の一覧は Attachment（A）のとおりである。

方法：

1. 提供された菌株 DSM4025 の育成

菌株に添付されていた菌株育成のプロトコールに従いアンプルを処理し凍結乾燥品を滅菌済みのハサミとピンセットを使い半分にした。
半分をアンプルに戻し、半分をその後の実験に用いた。
菌体を懸濁する際には Medium ではなく ddH_2O 200 μl を加えた。
菌体が解けにくかったため、同様に ddH_2O を更に 200 μl を加えた。
200 μl を NS2 培地に塗布した。
27℃で、4 日間培養を行った。

白金耳でプレート半分部分をなぞるように鈎菌し、dH_2O に溶かし込んだ。
A_{550} を測定。5 倍希釈で 0.3061 になるまで徐々に菌を希釈した。
12,000rpm、5 分にて菌体を回収した。

2. 染色体 DNA の調製

SepaGene（三光純薬株式会社）、DNeasy Tissue Kit（株式会社キアゲン）の 2 種類のキットを使用して染色体 DNA の調製を行った。
両方法ともキットに添付されているプロトコールに従って行った。
SepaGene は抽出法 I の手順にて行った。

SepaGene（抽出法Ⅰ）を使用したゲノムDNA抽出

1. A_{550}の測定値5倍希釈で0.3061となったものを　12,000rpm、5分の遠心を行い、集菌した。
2. 上清を除去した後、Tris-HCl（pH8.0）100uLを加え、均一に懸濁させた。
3. 室温で10分間静置した。
4. この懸濁液にチオシアン酸グアニジン100uLを加え、ピペットで緩やかに混和した。
5. クロロホルム50%を含む吸着剤700uLと、酢酸ナトリウム溶液400uLを加えた。
6. マイクロチューブのふたを閉め、乳濁化するまで10秒間上下に激しく振盪混和した。
7. 12,000rpm、15分間遠心した。
8. 核酸を含む上層を別のマイクロチューブに分取した。
9. 酢酸緩衝液を55uL加えた。
10. 605uLのイソプロピルアルコールを加え、軽く転倒混和した。
11. 12,000rpm、15分間遠心した。
12. 上清を静かに除去し、70%エタノール1mLを加え軽く転倒混和した。
13. 12,000rpm、15分間遠心した。
14. 上清を静かに除去し、核酸ペレットを軽く乾燥させた。

DNaseyを使用したゲノムDNA抽出

1. A_{550}の測定値5倍希釈で0.3061となったものを　12,000rpm、5分の遠心を行い、集菌した。
2. 180uLのATL　Bufferを加え懸濁した。
3. 20uLのProteinase Kを加え、混和し、細胞が完全に溶解するまで55℃でover　nightでインキュベートした。
4. 15秒間ボルテックスをかけた後、200uLのBuffer ALを加えよく混和した後、70℃で10分間インキュベートした。
5. 96－100%エタノールを200uL加え、十分に混和した。
6. 2mlのチューブにセットしたDNeasyカラムに5を静かにのせ、8000rpmで1分遠心を行い、ろ液を除去した。
7. DNeasyカラムを新たな2mlチューブにセットし、500uLのAW1 Bufferを加え、8000rpmで1分遠心し、ろ液を除去した。
8. DNeasyカラムを新たな2mlチューブにセットし、500uLのAW2

Buffer を加え 15,000rpm で 3 分間遠心し、DNeasy メンブレンを乾燥させ、ろ液を除去した。

9. DNeasy カラムを 2ml チューブにセットし、200uL の Buffer AE（10mM Tris-HCl, pH9.0. 0.5mM EDTA, pH9.0）を加えた後、室温で 1 分間静置した後、8000rpm で 1 分間遠心し、溶出させた。
10. ステップ 9 をもう一度繰り返した。

3. プライマー作成

サワディー・テクノロジーにて指示されたプライマーを作成した。
合成後、オリゴパックカラム（OPC）にて精製を行った。

解析 1 のプライマー

Forward: A697f	5'− TACgAAgCCC gTTggATgAC — 3'
Reverse: A1000r	5'− TCgggTTgAT CgACTgCAgA — 3'

解析 2 のプライマー

Forward: A"479f	5' − TATTCgACgT CgATCgCggT − 3'
Reverse: A"780r	5' − AACTgCTgAg gTgCCgTAgT − 3'

4. 目的領域の PCR

方法 3 で合成を行った 2 ペアのプライマーを使用し、PCR を行い、目的領域の増幅を行った。

Let's Go PCR	48.5μL
primer	0.5μL (20μM)
DNA	1 μL
	50 μL

90℃	1min	
↓	以下 35cycle 繰り返す	
95℃	30 sec	}
50℃	30 sec	}
68℃	30 sec	}
↓		
72℃	30 min	
4℃	ストック	

電気泳動にて増幅領域を確認後、目的領域を切り出し、High pure PCR Product Purification Kit（ロシュダイアグノーシス）にて精製を行った。

5. 塩基配列決定

方法4で増幅したPCR産物をABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit（Applied Biosystems Japan）のプロトコールに従い解析を行った。

Terminator Ready Reaction Mix	8.0μL
Template	50 ng
primer	4 pmol
DW	
total	20μL

PCR条件は以下の通り。

25cycle繰り返す		
96℃	10sec	}
50℃	5sec	}
60℃	4min	}

ABI Prism 377にセットし各サンプルを3μLアプライした。
overnightにて泳動を行った。

結果：

1. 染色体 DNA の調製

DNeasy で調製した DNA はステップ 10 のように、Buffer AE（10mM Tris-HCl, pH9.0, 0.5mM EDTA, pH9.0）100μL に溶解した。
SepaGene で調製した DNA は TE Buffer（10mM Tris-HCl, pH8.0. 1mM EDTA, pH8.0） 50μL に溶解し濃度を測定した。

```
DU530 S/N: 9706U3000073 1.03
31-AUG-00  10:30:36  NUCLEIC ACID  Double Ratio &Conc  Group 0315
PATHcm: 1.0000
```

Sample	Net A 260.0	Net A 280.0	Net A 230.0	260.0/ 280.0	260.0/ 230.0	
DNeasy 0001 ×20	0.030	0.015	0.014	2.041	2.180	DNeasy
DNeasy 0002 ×20	0.026	0.014	0.003	1.811	8.151	28ng/ml × 100ml
Sepa 0003 ×50	0.209	0.105	0.096	1.996	2.170	Sepagene
Sepa 0004 ×50	0.197	0.099	0.092	1.992	2.151	0.508ng/ul ×50ul

2. PCR

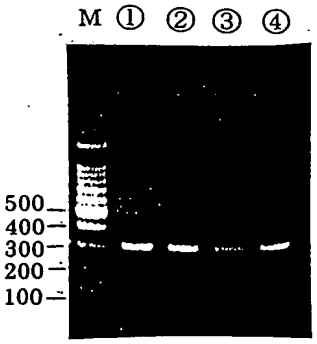
解析 1. 名称 Enzyme A

304bp

解析 2. 名称 Enzyme A"

302bp

電気泳動写真より目的の大きさの PCR 産物が得られている事が分かる。
2%アガロースゲル、PCR 産物は各 3μL を泳動した。



① EnzymeA(nt697-1000)
② EnzymeA(nt697-1000)
③ EnzymeA(nt479-780)
④ EnzymeA(nt479-780)

①③は template DNA に SW-2/DNeasy を使用
②④た template DNA に SW-2/Sepagene を使用

3. 塩基配列決定

指示通り、テンプレートに DNeasy にて抽出したゲノム DNA をもちいて、F／R のプライマーを用い両方向からシークエンス反応を行った。
シークエンス配列は Attachment　B の通り
波形データは　Attachment　C の通り

私たちは各々、この試験報告書が、株式会社キューリンにより私達に供給された *Gluconobacter　oxydans*　DSM 4025 の染色体 DNA から単離された増幅産物のクローニングと配列決定　（ Attachment B に示す ）についての真実で正確な記述であることを断言し、署名いたします。

日付 12. Sep. 2002　　株式会社　サワディー・テクノロジー

間下 正雄、営業部兼マーケティング部マネージャー

日付 13/Sep/2002

増尾 正則、運営管理者

Attachment (A)

使用試薬および使用機器

使用試薬

- NS2 培地（依頼者より提供）
- 核酸抽出剤 SepaGene （三光純薬株式会社）
- DNeasy Tissue Kit（株式会社 キアゲン）
- ABI Prism BigDye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems Japan)
- AmpliTaq DNA Polymerase (Applied Biosystems Japan)
- Let's Go PCR Kit （株式会社 サワディー・テクノロジー）
- High Pure PCR Purification Kit （ロシュダイアグノーシス）

使用機器

- サンヨーインキュベーター MIR153 （サンヨー）
- ABI prism 377 (Applied Biosystems Japan)
- GeneAmp PCR System 9600 (Applied Biosystems Japan)
- ミニサイクラー （MJ Reserch）
- MicroCen13D （Herolab）
- Mupid ミニゲル泳動槽 （Mupid）

Attachment (B)

Sequences of the amplified products.

39F903 (697-1000)/A697f.Seq

TTNCGTGCCT GGGGCCAGAT CACCTATGAC CCCGTCACCA ACCTTGTCCA
CTACGGCTCG ACCGCTGTGG GTCCGGCGTC GGAAACCCAA CGCGGCACCC
CGGGCGGCAC GCTGTACGGC ACGAACACCC GTTTCGCCGT GCGTCCTGAC
ACGGGCGAGA TTGTCTGGCG TCACCAGACC CTGCCCCGCG ACAACTGGGA
CCAGGAATGC ACGTTCGAGA TGATGGTCAC CAATGTGGAT GTCCAACCCT
CGACCGAGAT GGAAGGTCTG CAGTCGATCA ANCGAAANNN NNNNNNNNNN
NNNNN

41F903 (697-1000)/A1000r.Seq

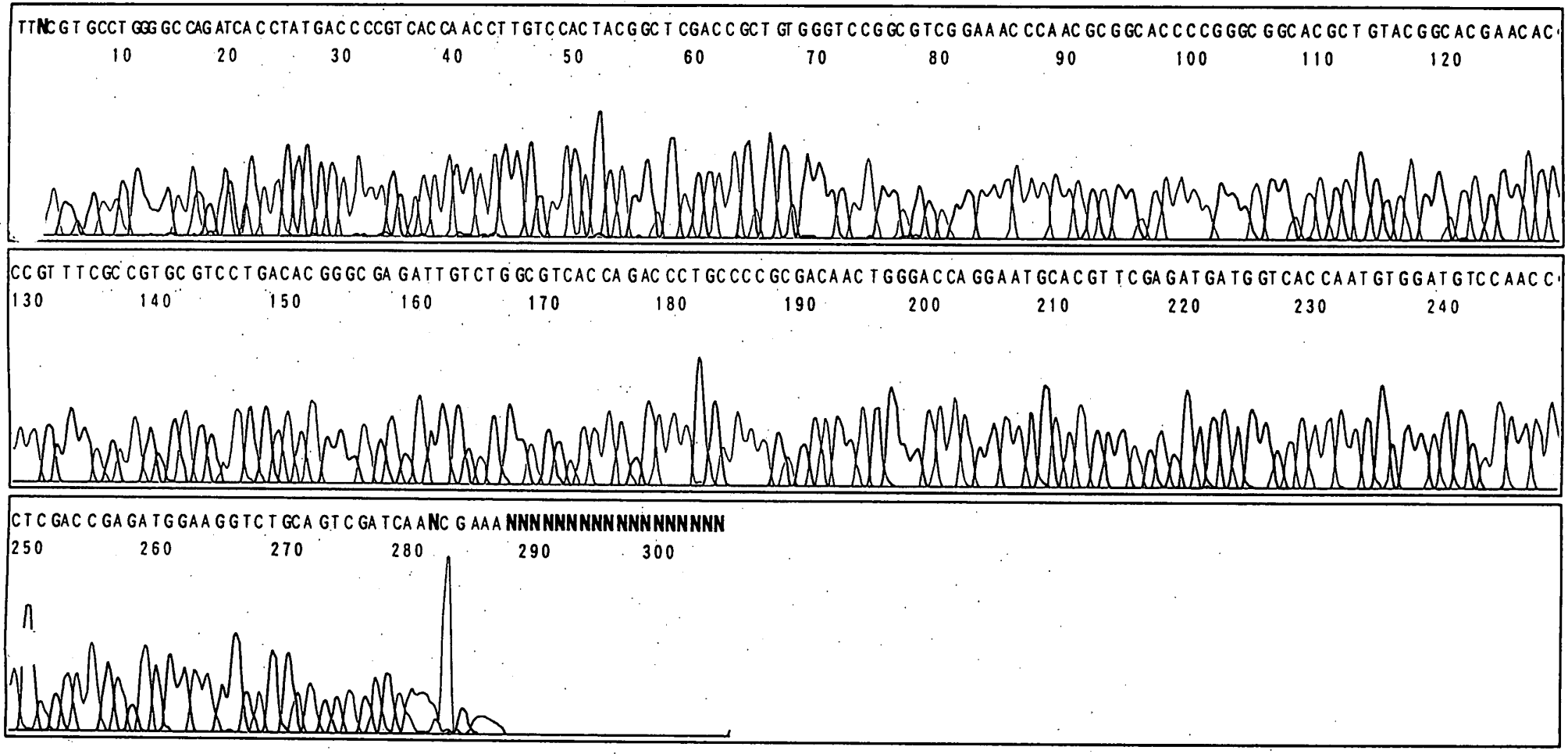
TTCCTCTTGG TCGAGGGTTG GACATCCACA TTGGTGACCA TCATCTCGAA
CGTGCATTCC TGGTCCCAGT TGTCGCGGGG CAGGGTCTGG TGACGCCAGA
CAATCTCGCC CGTGTCAGGA CGCACGGCGA AACGGGTGTT CGTGCCGTAC
AGCGTGCCGC CCGGGGTGCC GCGTTGGGTT TCCGACGCCG GACCCACAGC
GGTCGAGCCG TAGTGGACAA GGTTGGTGAC GGGGTCATAG GTGATCTGGC
CCCAGGCACC GGTCATCCAA CGGGCTTTGT AANNNNNNNN NNNNNNNNNN
N

43F903 (479-780)/A479f.Seq

AAAGCACTTT ATGGNCTCGA ACTCTCCGGC CCGATTGTCG CCAATGGCGT
CATCGTTGCG GGCTCGACCT GTCAGTATTC GCCGTTCGGC TGTTTCGTTT
CGGGCCACGA CTCGGCCACC GGTGAAGAGC TGTGGCGCAA CACCTTTATC
CCGCGCGCCG GCGAAGAGGG TGATGAGACC TGGGGCAATG ATTACGAGGC
CCGCTGGATG ACCGGCGTTT GGGGCCAGAT CACCTATGAC CCCGTTGGCG
GCCTTGTCCA CTACGGCACC TCAAGAGTTA ANANNNNNNN NNNNNNNNN

45F903 (479-780)/A780r.Seq

GACAAGGCTN NCACGGNGTC ATAGGTGATN TGGCCCCAAA CGCCGGTCAT
CCAGCGGGCC TCGTAATCAT TGCCCCAGGT CTCATCACCC TCTTCGCCGG
CGCGCGGGAT AAAGGTGTTG CGCCACAGCT CTTCACCGGT GGCCGAGTCG
TGGCCCGAAA CGAAACAGCC GAACGGCGAA TACTGACAGG TCGAGCCCGC
AACGATGACG CCATTGGCGA CAATCGGGCC GGACGAGTTC GAGACCATAT
CCGTGCCTTG ACCGCGATCG ACGTCCATAA ANNNNNNNNN NNNNNNNNNN


TTNCGTGCCTGGGGCCAGATCACCTATGACCCCGTCACCAACCTTGTCCACTACGGCTCGACCGCTGTGGGTCCGGCGTCGGAAACCCAACGCGGCACCCCGGGCGGCACGCTGTACGGCACGAACAC
10 20 30 40 50 60 70 80 90 100 110 120
CCGTTTCGCCGTGCGTCCTGACACGGGCGAGATTGTCTGGCGTCACCAGACCCTGCCCCGCGACAACTGGGACCAGGAATGCACGTTCGAGATGATGGTCACCAATGTGGATGTCCAACC
130 140 150 160 170 180 190 200 210 220 230 240
CTCGACCGAGATGGAAGGTCTGCAGTCGATCAANCGAAANNNNNNNNNNNNNNNNNN
250 260 270 280 290 300

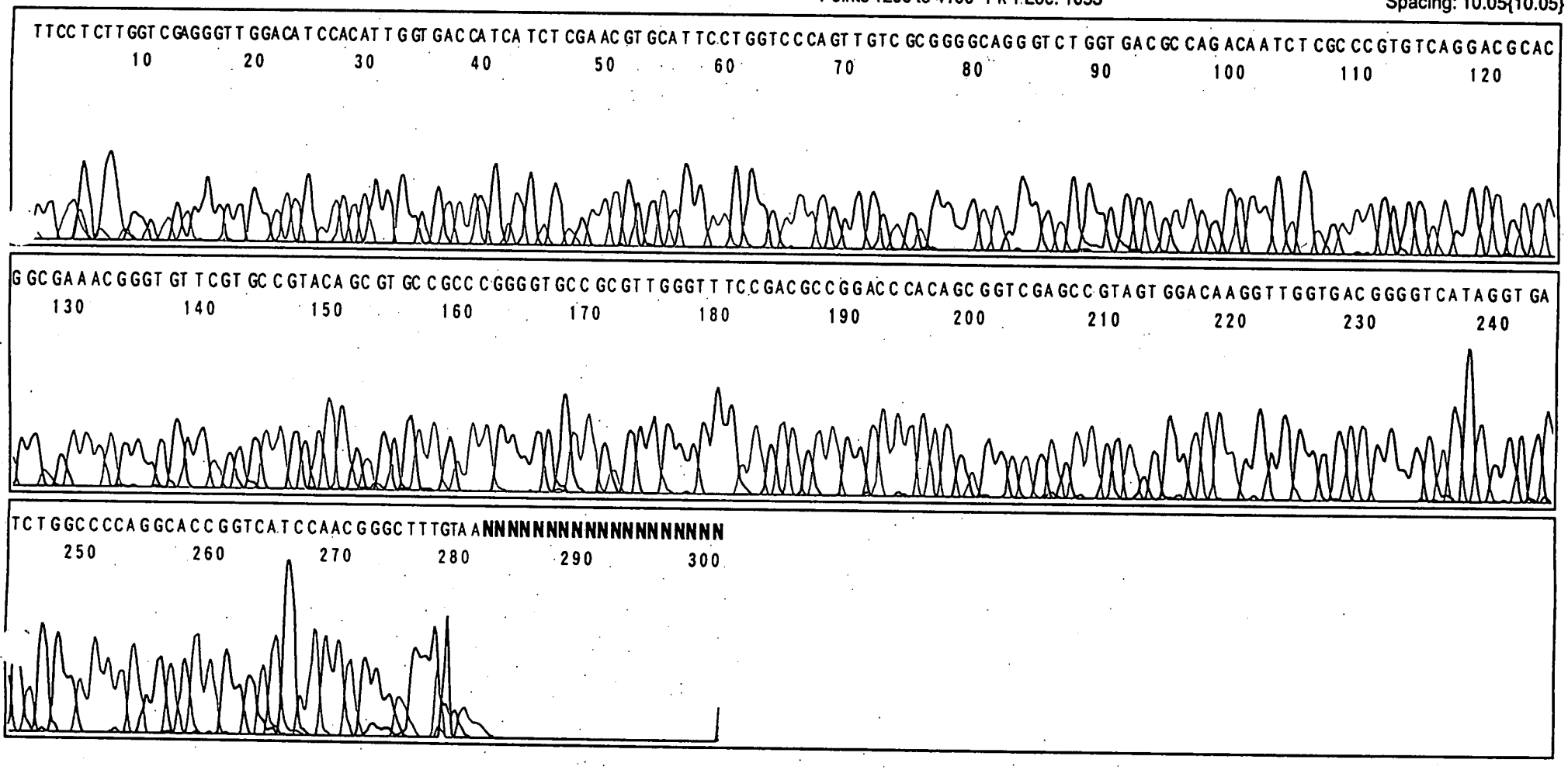
Model 377
Version 3.3
ABI100
Version 3.2

41•F903 (697-1000)/A1000r

F903 (697-1000)/A1000r
Lane 41

Signal G:1010 A:770 T:388 C:568
DT{BD Set Any-Primer}
dRhodamine Teminator Matrix
Points 1200 to 4100 Pk 1 Loc: 1053

Tue, Sep 5, 2000 2:03 PM
Mon, Sep 4, 2000 6:18 PM
Spacing: 10.05{10.05}



Attachment C-2

ABI PRISM

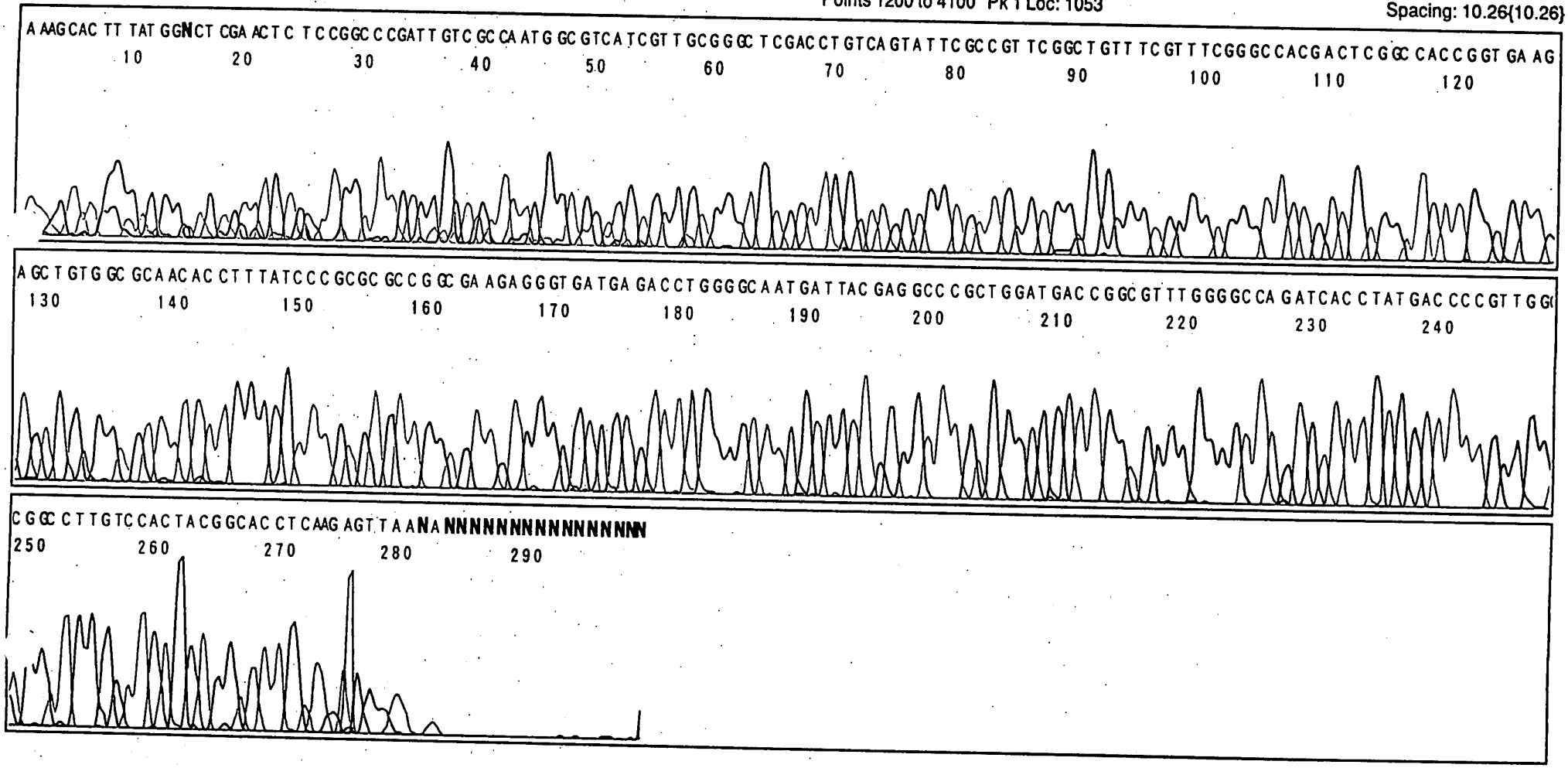
Model 377
Version 3.3
ABI100
Version 3.2

43•F903 (479-780)/A479f

F903 (479-780)/A479f
Lane 43

Signal G:523 A:379 T:211 C:222
DT {BD Set Any-Primer}
dRhodamine Teminator Matrix
Points 1200 to 4100 Pk 1 Loc: 1053

Tue, Sep 5, 2000 2:04 PM
Mon, Sep 4, 2000 6:18 PM
Spacing: 10.26{10.26}



Attachment C-2

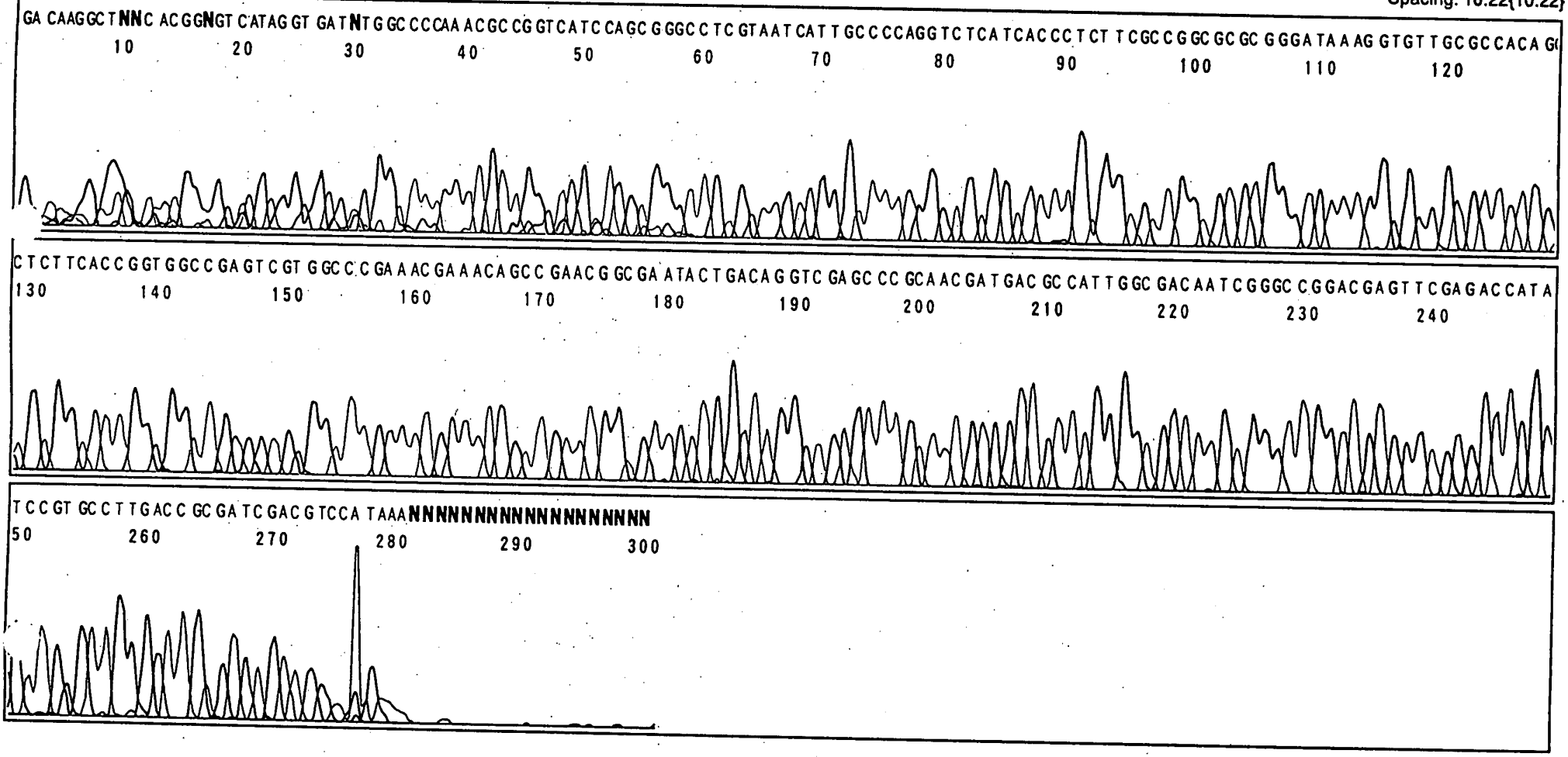
Model 377
Version 3.3
ABI100
Version 3.2

45•F903 (479-780)/A780r

F903 (479-780)/A780r
Lane 45

Signal G:658 A:610 T:254 C:336
DT{BD Set Any-Primer}
dRhodamine Teminator Matrix
Points 1200 to 4100 Pk 1 Loc: 1053

Tue, Sep 5, 2000 2:04 PM
Mon, Sep 4, 2000 6:18 PM
Spacing: 10.22{10.22}



Attachment C-1

(Translation)

Experimental Report

Experiment Number: F-903

Theme: Sequencing of the genes of the strain DSM 4025

Date: September 12, 2002

Name of Client:

Nippon Roche Research Center, Nippon Roche K.K. (NRKK)
Kajiwara 200, Kamakura-shi, Kanagawa-ken, 247-8530, Japan

K.K. Sawady Technology
1-29-10, Maeno-cho, Itabashi-ku, Tokyo, 174-0063, Japan

Experimental Report

Theme: Sequencing of the genes of the strain DSM 4025

Experiment Number: F-903 (SW-002)

Requester of the Experiment:

Nippon Roche Research Center, Nippon Roche K.K.
Kajiwara 200, Kamakura-shi, Kanagawa-ken, 247-8530, Japan

Experimenter (company):

K.K. Sawady Technology
1-29-10, Maeno-cho, Itabashi-ku, Tokyo, 174-0063, Japan
Name of COO: Masanori Masuo

Person responsible for the Experiment:

Name: Masao Mashita
Company: K.K. Sawady Technology
Name: Yoshitaka Murata
Company: K.K. Kyurin

Persons responsible for performing the Experiment:

Name: Susumu Yamashita
Company: K.K. Sawady Technology

Name: Masako Nomaguchi
Company: K.K. Kyurin

Term of the Experiment:

Initiated on: August 2000
Finished on: September 2000
Date of final report: September 12, 2002

Sample:

Lyophilized strain in an ampoule
Supplied by Nippon Roche Research Center, Nippon Roche K.K.

Reagents and Devices:

The reagents and the instruments used for performing the present Experiment are listed in Attachment (A).

Methodology:

1. Cultivation of the strain DSM 4025 supplied:

The ampoule was treated in accordance with the protocol for the cultivation of the strain attached to the ampoule, wherein the lyophilized material was cut into two equal pieces with a sterilized pair of scissors and pincette. One piece was returned to the ampoule and the other was used for the experiment.

For suspending the cells, 200 µl of ddH_2O was added instead of a medium. As the strain was hard to be suspended, an additional 200 µl of ddH_2O was added. 200 µl of the suspension was added to NS2 medium. The medium was incubated at 27°C for 4 days.

Cells from a half of the plate were scraped with a platinum loop (to collect the strain) to be dissolved in dH_2O. The cell suspension was gradually diluted to A_{550} of 0.3061 when diluted by 5 fold. The cells of the strain were collected by centrifugation at 12,000 rpm for 5 minutes.

2. Preparation of chromosomal DNA

The chromosomal DNA was prepared using two commercial kits, which are SepaGene (Sankou Junyaku K.K.) and DNeasy Tissue Kit (K.K. Qiagen). Both the kits were used in accordance with the protocols attached to the kits. With respect to SepaGene, Extraction Method I was used.

Extraction of genome DNA with SepaGene (Extraction Method I)

1. Collected the cells from the cell suspension of A550 = 0.3061 obtained by 5-fold dilution of above mentioned cell suspension by centrifugation at 12,000 rpm for 5 minutes.
2. After discarding the supernatant, the cells were suspended homogeneously by adding 100 µl of Tris-HCl (pH8.0).

3. The cells were maintained at room temperature for 10 minutes.
4. Added 100 μl of guanidine-thiocyanate into the cell suspension and mixed the solution gently with a pipette.
5. Added the 700 μl absorbant containing 50% chloroform and 400 μl of sodium acetate.
6. Closed the tube and mixed vigorously until the solution became emulsion for 10 seconds.
7. Centrifuged at 12,000 rpm for 15 minutes.
8. Took the upper phase containing nucleic acids.
9. Added 55 μl of acetate buffer.
10. Added 605 μl of isopropyl alcohol and mixed gently.
11. Centrifuged at 12,000 rpm for 15 minutes.
12. Discarded the supernatant, added 1 ml of 70% ethanol and mixed gently.
13. Centrifuged at 12,000 rpm for 15 minutes.
14. Discarded the supernatant and dried the nucleic acid pellet briefly.

Extraction of genome DNA with DNeasy (Extraction Method II)

1. Collected the cells from the cell suspension of A550 = 0.3061 obtained by 5-fold dilution of the above-mentioned cell suspension by centrifugation at 12,000 rpm for 5 minutes.
2. Added 180 μl ATL Buffer and suspended the cells.
3. Added 20 μl of Proteinase K, mixed the suspension and incubated at 55°C for 1 overnight until the cells were completely lysed.
4. Vortexed for 15 seconds, added 200 μl AL Buffer, mixed well, and incubated the suspension at 70°C for 10 minutes.
5. Added 96 to 100% ethanol and mixed well.
6. Loaded the solution obtained in step 5 gently onto a DNeasy column set on a 2 ml tube, centrifuged it at 8,000 rpm for 1 minute, and discarded the filtrate.
7. Set the DNeasy column onto a new 2 ml tube, added 500

µl AW1 Buffer, centrifuged it at 8,000 rpm for 1 minute, and discarded the filtrate.

8. Set the DNeasy column onto a new 2 ml tube, added 500 µl AW2 Buffer, centrifuged it at 15,000 rpm for 3 minutes, dried the membrane of DNeasy and discarded the filtrate.
9. Set the DNeasy column onto a new 2 ml tube, added 200 ml AE Buffer consisting of 10 mM Tris-HCl, pH 9.0, 0.5 mM EDTA, kept the solution at room temperature for 1 minute, centrifuged it at 8,000 rpm for 1 minute and eluted the solution.
10. Repeated the step 9.

3. Preparation of primers

The primers requested by NRKK were prepared by Sawady Technology. After the synthesis, they were purified through an Oligopack column (OPC).

Primers for Analysis 1:

Forward: A697f	5'- TACGAAGCCC GTTGGATGAC -3'
Reverse: A1000r	5'- TCGGGTTGAT CGACTGCAGA -3'

Primers for Analysis 2:

Forward: A"479f	5'- TATTCGACGT CGATCGCGGT -3'
Reverse: A"780r	5'- AACTGCTGAG GTGCCGTAGT -3'

4. PCR of the target region

Two PCRs were performed using the two pairs of primers synthesized according to the above method 3, respectively to amplify the targeted regions.

Let's Go PCR	48.5 µL
Primers	0.5 µL (20 µM)
DNA	1 µL
	50 µL

90°C 1 min.
↓ 35 cycles of the following 3 steps were repeated
95°C 30 sec.
50°C 30 sec.
68°C 30 sec.
↓
72°C 30 min.
4°C for stock

After confirming the amplified regions by electrophoresis, the desired regions were cut out and purified using a High Pure PCR Product Purification Kit (Roche Diagnostics).

5. Determination of the nucleotide sequences

The PCR products amplified in the above method 4 were analyzed by an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan) in accordance with the protocol supplied by the manufacturer as explained briefly below.

Terminator Ready Reaction Mix	8.0 μL
Template	50 ng
Primer	4 pmol
dH2O	balance to 20 μL
Total	20 μL

PCR condition is as follows:

25 cycles of the following steps
96°C 10 sec
50°C 5 sec
60°C 4 min

3 μL of each sample was applied to an ABI Prism 377 apparatus. The electrophoresis was run overnight.

Results:

1. Preparation of chromosomal DNA

The DNA prepared using DNeasy was dissolved in 100 μL of AE Buffer consisting of 10 mM Tris-HCl, pH 9.0, 0.5 mM EDTA, and the DNA prepared by using SepaGene was dissolved in 50 μL of TE Buffer consisting of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, which were measured for the concentrations.

[Data will be pasted.]

2. PCR

Analysis 1: designated as Enzyme A

304 bp

Analysis 2: designated as Enzyme A"

302 bp

The electrophoresis pattern revealed that the PCR products having the target sizes were obtained.

2% agarose gel and each 3 μL of the PCR products were used for the electrophoresis.

[Picture of electrophoresis gel will be pasted.]

3. Determination of the nucleotide sequences

In accordance with the request, the respective samples were used for bidirectional sequencing (from both the direction) using forward and reverse primers.

The sequences determined are as described in Attachment (B):

The chromatograms are also attached hereto as Attachment (C).

We the undersigned each affirm that this Experimental Report is a true and accurate description of the cloning and sequencing of the amplified products set forth in Attachment B, which products were isolated from the chromosomal DNA of *Gluconobacter oxydans* DSM 4025 supplied to us by K.K. Kyurin

Dated:_____ For: K.K. Sawady Technology Co. Ltd.

By: _____

Masao Mashita, Sales & Marketing Director

Dated: _____ By: _____

日付

Masanori Masuo, COO

Attachment (A)

Reagents

- NS2 medium (supplied by the Requester)
- Reagent for extracting nucleic acids, SepaGene (Sankou Junyaku K.K)
- DNeasy Tissue Kit (K.K. Qiagen)
- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan)
- AmpliTaq DNA Polymerase (Applied Biosystems Japan)
- Let's Go PCR Kit (K.K. Sawady Technology)
- High Pure PCR Purification Kit (Roche Diagnostics K.K.)

Instruments

- Sanyo Incubator MIR153 (Sanyo)
- ABI Prism 377 (Applied Biosystems Japan)
- GeneAmp PCR System 9600 (Applied Biosystems Japan)
- Minicycler (MJ Research)
- MicroCen 13D (Herolab)
- Mupid mini-gel electrophoresis apparatus (Mupid)

Attachment (B)

Sequences of the amplified products.

39F903 (697-1000)/A697f.Seq

TTNCGTGCCT GGGGCCAGAT CACCTATGAC CCCGTCACCA ACCTTGTCCA
CTACGGCTCG ACCGCTGTGG GTCCGGCGTC GGAAACCCAA CGCGGCACCC
CGGGCGGCAC GCTGTACGGC ACGAACACCC GTTTCGCCGT GCGTCCTGAC
ACGGGCGAGA TTGTCTGGCG TCACCAGACC CTGCCCCGCG ACAACTGGGA
CCAGGAATGC ACGTTCGAGA TGATGGTCAC CAATGTGGAT GTCCAACCCT
CGACCGAGAT GGAAGGTCTG CAGTCGATCA ANCGAAANNN NNNNNNNNNN
NNNNN

41F903 (697-1000)/A1000r.Seq

TTCCTCTTGG TCGAGGGTTG GACATCCACA TTGGTGACCA TCATCTCGAA
CGTGCATTCC TGGTCCCAGT TGTCGCGGGG CAGGGTCTGG TGACGCCAGA
CAATCTCGCC CGTGTCAGGA CGCACGGCGA AACGGGTGTT CGTGCCGTAC
AGCGTGCCGC CCGGGGTGCC GCGTTGGGTT TCCGACGCCG GACCCACAGC
GGTCGAGCCG TAGTGGACAA GGTTGGTGAC GGGGTCATAG GTGATCTGGC
CCCAGGCACC GGTCATCCAA CGGGCTTTGT AANNNNNNNN NNNNNNNNNN
N

43F903 (479-780)/A479f.Seq

AAAGCACTTT ATGGNCTCGA ACTCTCCGGC CCGATTGTCG CCAATGGCGT
CATCGTTGCG GGCTCGACCT GTCAGTATTC GCCGTTCGGC TGTTTCGTTT
CGGGCCACGA CTCGGCCACC GGTGAAGAGC TGTGGCGCAA CACCTTTATC
CCGCGCGCCG GCGAAGAGGG TGATGAGACC TGGGGCAATG ATTACGAGGC
CCGCTGGATG ACCGGCGTTT GGGGCCAGAT CACCTATGAC CCCGTTGGCG
GCCTTGTCCA CTACGGCACC TCAAGAGTTA ANANNNNNNN NNNNNNNNN

45F903 (479-780)/A780r.Seq

GACAAGGCTN NCACGGNGTC ATAGGTGATN TGGCCCCAAA CGCCGGTCAT
CCAGCGGGCC TCGTAATCAT TGCCCCAGGT CTCATCACCC TCTTCGCCGG
CGCGCGGGAT AAAGGTGTTG CGCCACAGCT CTTCACCGGT GGCCGAGTCG
TGGCCCGAAA CGAAACAGCC GAACGGCGAA TACTGACAGG TCGAGCCCGC
AACGATGACG CCATTGGCGA CAATCGGGCC GGACGAGTTC GAGACCATAT
CCGTGCCTTG ACCGCGATCG ACGTCCATAA ANNNNNNNNN NNNNNNNNNN

Attachment (C)

[Chromatograms to be pasted.]